Phenylketonuria: optimizing care

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CHAPTER 6
Micronutrients, fatty acids and bone health in Phenylketonuria

Submitted

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Abstract

Introduction: In phenylketonuria the natural protein restricted diet, supplemented with micronutrient fortified amino acid mixtures, prevents severe cognitive impairment due to high phenylalanine levels. Nutrient deficiencies may occur as a result of the dietary restrictions. We aimed to evaluate intake and blood levels of micronutrients and essential fatty acids (FA), bone mineral density (BMD), bone turnover markers (BTM) and fracture history.

Methods: Sixty early diagnosed phenylketonuria patients (aged ≥1 year) were included in a multi-center cross sectional study. We assessed micronutrients, FA and BTM in blood, dietary intake and fracture history using a questionnaire, and BMD retrospectively.

Results: Dietary intake and serum levels of selenium (14 and 46% of patients) and 25-OH vitamin D2+3 (20 and 14% of patients) were inadequate. Zinc serum levels were below normal in 14% of patients despite adequate intake. Folic acid serum and intake levels were above normal. Despite safe total protein and fat intake, arginine plasma levels and erythrocyte eicosapentaenoic acid were below reference values in 19% and 6% of patients respectively. Low BMD (Z-score < -2) was slightly more prevalent in patients but lifetime fracture prevalence was comparable to the general population. Both resorption and formation BTM were elevated.

Conclusions: In general, patients with phenylketonuria have a normal nutrient status. However, the risk of low intake and blood levels of zinc, selenium, 25-OH vitamin D2+3, arginine, eicosapentaenoic acid, as well as observed high levels of folic acid, need attention. Fracture prevalence is normal but a slightly more prevalent low BMD and elevated BTM warrant further investigation.
INTRODUCTION

Phenylketonuria (PKU; MIM 261600) is an autosomal recessive disorder of phenylalanine (Phe) metabolism caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1), leading to severe cognitive impairment due to accumulation of Phe in the brain. With the introduction of newborn screening and the early institution of dietary treatment, cognitive impairment caused by PKU has nearly been eliminated in developed countries [1]. Based on the level of blood Phe at diagnosis, disease severity is classified as either classic or severe PKU (≥ 1200 μmol/L), mild to moderate PKU (600–1200 μmol/L), or mild hyperphenylalaninemia (mHPA; 360–600 μmol/L). Treatment consists of dietary Phe intake restriction (an essential amino acid (AA)) through a low protein diet in order to achieve safe Phe blood levels. Severely affected patients tolerate <500 mg of Phe, which is <10 grams of natural protein per day [2,3]. In order to guarantee a sufficient intake of daily protein, patients use designated amino acid mixtures (AAM) containing most amino acids (not Phe), vitamins and other micronutrients. Many different AAM are available with a highly variable composition of nutrients. In some AAM, calculation of the amount of added nutrients is based on needed daily calories and in others on advised intakes of protein per kilogram bodyweight [4]. Few studies evaluated intake and deficiencies of nutrients in patients with PKU and both normal and reduced intakes and blood levels of nutrients have been reported [5,6]. A minority of patients is responsive to treatment with tetrahydrobiopterin (BH4), a cofactor of PAH, which increases dietary Phe tolerance and thus permits relaxation of the diet [7]. Because BH4 has only recently (2009) become available for PKU treatment in the Netherlands, responsive patients on a relaxed diet find it difficult to make healthy food choices after years on a strict diet and deficiencies have been reported [6].

Furthermore, a decreased BMD in PKU has been frequently reported, although a recent systematic review showed that the prevalence of osteoporosis according to internationally accepted standards is low [8]. The relationship between bone health and (micro)nutrient deficiencies is insufficiently studied. In order to optimize treatment,
and to prevent deficiencies or potential toxic concentrations of micronutrients, there is a need for more insight into the nutrient intake and blood levels of patients with PKU [4]. The main objective of this study was to evaluate the intake of micronutrients and essential FA from natural protein containing food and AAM in patients with PKU, and to investigate the association between intake and blood levels of these micronutrients and essential FA. The secondary objectives were to investigate BMD, bone turnover markers (BTM) and fracture history in patients with PKU, and their associations with blood levels and intake of micronutrients and essential FA.
METHODS

Study Design

This cross-sectional multicenter study was performed in three Dutch metabolic centers (Amsterdam, Groningen and Maastricht) between May 2013 and May 2014. Inclusion criteria were: PKU diagnosed through newborn screening; age ≥1 year; continuous treatment with either a protein restricted diet with the use of an AAM, a protein restricted diet with AAM in combination with BH4 treatment or BH4 treatment without dietary protein restriction. Exclusion criteria were: changes in AAM in the month before inclusion and (planned) pregnancy.

Micronutrients, essential FA and BTM were assessed in blood after at least three hours fasting. An investigator-designed-questionnaire was used to evaluate daily dietary intakes, fracture history and amount of physical activity (sports, walking and cycling in the past year). Medication (including BH4) and dietary intake of AAM, natural protein containing food sources and supplementary vitamins/minerals were assessed. Patient reported intake was compared to the most recent dietary prescription from their treating dietician[9]. Patient records were studied to obtain Phe levels from dried blood spots over the last 12 months, and BMD Z-scores from dual-energy X-ray absorptiometry scans (DXA) performed between two years before to 6 months after inclusion. The study protocol was approved by the Ethics Committee of the AMC and patients/parents provided informed consent before participation.

Laboratory measurements

Results from collected blood specimens were all obtained from centralised laboratories, except for plasma amino acids which were assessed at the medical centre where the patient was under treatment. However, all three centres are certified by the ERNDIMQA, quality control for amino acid measurements (http://cms.erndimqa.nl/Control-Materials.aspx), and for this reason assessments are
easily comparable. Chemical analyses were performed at the Clinical Chemical Laboratory of the AMC; Fatty acid (FA) assessments were performed at the Laboratory Genetic Metabolic Disease of the AMC; Bone turnover markers (BTM) were provided by the Vrije Universiteit medical centre (VUmc) endocrinology laboratory.

**Analysis of micronutrients**

Analysis of micronutrients were performed after serum centrifugation at 2000 x g and being stored at -20°C until analysis. Roche diagnostics equipment was used to analyse *albumin* and *calcium* (the spectophometry method), *sodium* and *potassium* (the ion selective electrode assay indirect method), *magnesium* (the colorimetric method), *phosphate* (the molybdate reduction method), *transferrin* (the immunotubidimetry method) and *folic acid* and *vitamin B12* (the electrochemiluminescence immunoassay method). Atomic absorption spectroscopy using Beun de Rionde B.V. equipment was used to analyse *zinc*, and *selenium* measurements were performed using the Zeeman atomaire absorptionspectofometry. 25OH vitamin D2 + D3 was assessed with Liaison Diasorin equipment using the competitive chemiluminescence immunoassay. Finally, full blood *vitamin B1 and B6* were evaluated with HPLC Gynkotek Dionex equipment using reverse phase HPLC fluorimetric detection.

**Analysis of total Fatty acids**

We measured erythrocyte FA. Erythrocytes of venous EDTA blood were washed three times in isotonic saline, counted by routine hemocytometric analysis and frozen overnight in a BHT (2,6-di-tert-butyl-4-methylphenol)-coated eppendorf cup. Fifty microliters of the resulting hemolysate –for plasma also 50 μl– was transmethylated in 1 ml 3 M HCl by incubating for 4 hours at 90°C in the presence of 10 nmol internal standard; the methyl ester of 18-methylnonadecanoic acid. After cooling, the aqueous
layer was extracted in 2 ml hexane, and this extract was taken to dryness under nitrogen flow and resuspended in 80 μl of hexane. One microliter of this solution was injected into a Hewlett Packard GC 5890 equipped with an Agilent J&W HP-FFAP, 25m, 0.20mm, 0.33µm GC Column and eluting fatty acid methylesters were detected by flame ionization detection. Fatty acid concentration were calculated using the known amount of internal standard and expressed as pmol/10^6 cells for erythrocytes and in μM for plasma.

**Analysis of plasma amino acids**

To assess amino acids plasma was prepared from heparinized blood by centrifugation at 1000 x g and stored at -20°C until analysis. Plasma amino acids were analysed using the JEOL AminoTac amino acid analyser JLC-500/V according to the manufacturer’s instructions.

**Bone Mass Density measurements**

Hologic Discovery imaging equipment was used with reference data from the general Dutch population to compare patient outcomes to [10]. According to the Society for Clinical Densitometry (ISCD) a diagnosis of osteoporosis in children, men and premenopausal women is based on a BMD Z-score below -2 coupled with a significant fracture history (two or more long bone fractures by age ten years and/or three or more long bone fractures at any age up to nineteen years, or at least one vertebral compression fractures in the absence of trauma) [11].

**Dietary intake and growth parameters**

Weight and height were collected, and age and gender appropriate Z-scores were calculated based on Dutch population references [12]. From the questionnaire the daily intake of micronutrients, protein and fat was calculated. Results were compared to the
recommended daily dietary allowance provided by the Federal Public Service Health of Belgium (basing its recommendations on previously performed research in the Dutch population and on recommendations of the USA [13]), and the safe advised range (SAR) of intake as recommended by the European Food Safety Authority (EFA) [14]. The recommended dietary allowance is based on +2 SD of the required dietary intake and thus provides sufficient intakes for 97.5% of the general population [15].

**Statistical analysis**

For all analyses the Statistical Package for Social Sciences (SPSS) Windows version 19 was used. Descriptive statistics were used to assess intakes and blood levels of micronutrients, AA, FA, BMD Z-scores and BTM. Concerning laboratory and dietary intake levels, we decided that levels outside of the reference range are of interest to study (remarkable). The reference range provides mean ± 2 SD for a healthy age matched population, hence remarkable results represent levels below the P5 or above P95 of normal. Results are reported as medians and displayed in scatterplots in which reference ranges are indicated; data are shown separated in age groups equal to available age groups used to indicate reference ranges. In addition, Mann–Whitney U (not normally distributed continues variables) or Chi square tests (nominal data) were conducted to test differences between patients based on disease severity, BH4 and supplement (not AAM) use.

Multivariable linear regression analyses was intended to investigate associations between blood levels, BMD, BTM and intake.
RESULTS

Participants
Sixty out of 102 eligible patients (58.8 %) agreed to participate in the study (43/51 Amsterdam, 10/32 Groningen and 7/19 Maastricht). The main reason for refusal was time constraints of patients. One enrolled patient was not included in the dietary analyses, because answers given in the questionnaire compared to the prescribed intake were unequal. None of the participants had restricted mobility, or used medication affecting bone status. Patient characteristics are displayed in Table 1.

Protein intake, phenylalanine levels and BH4 use
Detailed information on protein intake and Phe levels is shown in table 1. All patients had a total protein intake above minimal safe recommendations. Twenty-four percent of patients had a Phe tolerance of <500 mg per day (severe phenotype). BH4 was used by 14 patients; 10/14 additionally treated with natural protein restriction and AAM, 4/14 were off diet. Based on coincidence and taste preferences twenty-five different AAM were used, most frequently Milupa PKU-2-prima (n=11), Milupa PKU-2-mix (n=10), Vitafluo PKU cooler (n=9) and Milupa PKU-3-advanta (n=6).

The median percentage of Phe measurements above recommended range in the year before inclusion varied from 33-60%, increasing with age (Table 1); comparable to findings in other cohorts of PKU patients [16,17].

Laboratory results

Laboratory results of two patients were incomplete: in one AA were not evaluated and in the other only vitamins and FA were measured. The laboratories assessing AA made different choices on AA measured in routine PKU follow up, and therefore the total
number of assessed samples differs. We report on remarkable differences when compared to reference ranges that might have clinical implications.

**Table 1.** Patient characteristics

<table>
<thead>
<tr>
<th>Population</th>
<th>Age in years</th>
<th>Male</th>
<th>BMI SD for age and sex (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>13.0 (6 – 17)</td>
<td>25 (41.7)</td>
<td>0.45 (-0.18 - 1.24)</td>
</tr>
<tr>
<td>1 - 11</td>
<td>6.0 (4.5-9)</td>
<td>10 (40)</td>
<td>0.45 (0.08 - 0.96)</td>
</tr>
<tr>
<td>12 - 17</td>
<td>15 (13.3-15.8)</td>
<td>10 (50)</td>
<td>0.42 (-0.49 - 1.06)</td>
</tr>
<tr>
<td>18 – 39</td>
<td>29 (20.8-35.8)</td>
<td>5 (33.3)</td>
<td>0.49 (-0.40 - 2.65)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein intake in g/day</th>
<th>n</th>
<th>Total protein intake median (IQR)</th>
<th>Prot. natural sources median (IQR)</th>
<th>n</th>
<th>Prot. amino-acid supplement median (IQR)</th>
<th>BH4 use n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>59</td>
<td>64.8 (43.1-76.6)</td>
<td>14.6 (10.1 – 26.4)</td>
<td>55</td>
<td>45.00 (25.2 – 62.2)</td>
<td>14 (24)</td>
</tr>
<tr>
<td>1 - 11</td>
<td>24</td>
<td>39.7 (34.5-58.1)</td>
<td>10.8 (7.9-25.4)</td>
<td>23</td>
<td>25.2 (15.2-39.0)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>12 - 17</td>
<td>20</td>
<td>74.7 (63.9-83.5)</td>
<td>16.3 (12.0-27.3)</td>
<td>19</td>
<td>60.0 (40.0-63.0)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>18 - 39</td>
<td>15</td>
<td>81.3 (72.5-86.1)</td>
<td>19.0 (12.5-28.2)</td>
<td>13</td>
<td>60 (50.5-71.7)</td>
<td>2 (14)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenylalanine</th>
<th>n</th>
<th>Plasma Phe values (µmol/L) median (IQR)</th>
<th>% of bloodspot Phe values above range per patient median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 11</td>
<td>24</td>
<td>302 (193 – 342)</td>
<td>33 (0 – 67.5)</td>
</tr>
<tr>
<td>12 - 17</td>
<td>20</td>
<td>611 (401 – 778)</td>
<td>57 (0 – 100)</td>
</tr>
<tr>
<td>18 - 39</td>
<td>15</td>
<td>804 (522 – 978)</td>
<td>60 (0 – 100)</td>
</tr>
</tbody>
</table>
Micronutrients

Remarkable dietary results are reported in Figure 1 and 2, blood levels in Figure 3 and 4. The intake of vitamin D was below the advised minimum intake of 5 μg/day [18] in 12/59 patients. Vitamin D supplements were used in 12/60 patients. The 25-OH vitamin D2+3 serum level was below the reference range of 50 nmol/L in 8/59 patients and below 25 nmol/L in 2 of them. The lowest serum levels were found in one patient using AAM and in four patients treated with BH4 off diet. No significant differences in serum levels were found between supplemented and un-supplemented patients for vitamin D (72 vs 70 nmol/L).

Daily selenium intakes varied from 5-125 μg/day, and 27 patients had serum levels below the reference range (7/27 using BH4 with AAM). Selenium supplements were used by 6/60 patients. No significant differences in serum levels were found between supplemented and un-supplemented patients for selenium (0.87 vs 0.80 μmol/L).

Despite dietary zinc intake being well above the norm (48/59 patients), serum levels below the reference range were found in 8/59 patients (none using BH4). Severe patients showed significantly higher median zinc levels than mild patients: 12.5 vs 10.6 μmol/L.

Intakes of folic acid, magnesium, vitamin B6 and B12 were above the advised range in our patients. Folic acid intake was above SAR in 5 patients and 26/56 patients showed serum levels above reference range. Patients used the following supplements: magnesium (n=1), multivitamin tablets (n=4), vitamin B complex (n=1). Blood levels above reference were also found for magnesium (9/59 ), vitamin B6 (52/60) and vitamin B12 (11/58). Patients off diet treated with BH4 did not show elevated magnesium and folic acid levels (n=5). Severe patients showed significantly higher levels of vitamin B12 (median 600 vs 482 pmol/L). No significant differences were found between supplemented and un-supplemented patients for the micronutrients folic acid, magnesium, vitamin B12 and B6.
**Figure 1.** Dietary intake of vitamin D, selenium, zinc and folic acid

Median, minimum and maximum values compared to the recommended daily dietary allowance. Safe Advised Range (SAR) values:

- **SAR for vitamin D**: 100 µg/day
- **SAR for selenium ages ≥14 years**: 250 µg/day
Figure 2. Dietary intake magnesium, vitamin B6, vitamin B12 and fat

Median, minimum and maximum  
N = patients  m = male  f = female  
Safe Advised Range (SAR)  
SAR for vitamin B6 ≥4 years = 7 mg/day
Figure 3. Blood levels of 25-OH vitamin D2+3, selenium, zinc and folic acid

Median, minimum and maximum  

Reference range  

N = patients
Figure 4. Blood levels of vitamin B6, vitamin B12 and magnesium

Median, minimum and maximum  

Reference range  

N = patients
Amino acids

Plasma levels were below reference ranges for asparagine (22/59), 2-aminobutyric acid (10/50), tyrosine (13/59) and arginine (33/58). Hydroxyproline plasma levels were elevated (11/40) and ornithine levels normal to high (16/59) (Figure 5). Phe levels were above reference range in all ages. Low asparagine was significantly more often found in patients not using BH4 (2/15 patients using BH4 versus 20/44 not using BH4).

Erythrocyte fatty acids

In 57/59 patients the daily total fat intake of patients was below 40% of the total caloric intake (SAR) and for 34 patients below the minimal recommended 20% (Figure 1). Of 55 patients treated with AAM 35 used essential FA containing AAM and none used additional FA supplements.

Patients had total erythrocyte FA levels within reference range. Levels of the essential FA linoleic (C18:2ω6, LA) and α-linolenic acid (C18:3ω3, ALA) were unremarkable. The following metabolite levels of these FA were remarkable: 10/60 patients showed lowered levels for eicosapentaenoic acid (C20:5ω3, EPA), and elevated levels were found in 11/60 in homo-γ-linolenic acid (C20:3ω6), 23/60 in docosatetraenoic acid (C22:4ω6), 26/60 in docosapentaenoic acid (C22:5ω6), and 10/60 in docosahexaenoic acid (DHA; c22:6ω3) (Figure 6).

Median DHA and EPA levels were higher both in patients using FA supplemented AAM and in patients without restriction of natural protein, when compared to patients using AAM without FA supplementation (DHA 23 and 22 vs 16.5 pmol/10E6 cells; EPA 2.7 and 2.4 vs 2.1 pmol/10E6 cells).
**Figure 5.** Plasma amino acids

Median, minimum and maximum  
N = patients

Tyrosine data points:
patients that fasted overnight □  
patients that did not fast overnight ▲

Reference range

N = 3  N = 17  N = 19  N = 12

N = 3  N = 17  N = 24  N = 15

N = 3  N = 17  N = 23  N = 15

N = 3  N = 17  N = 24  N = 15

N = 3  N = 17  N = 24  N = 8
Figure 6. Essential fatty acids: α-linolenic acid and metabolites

Median, minimum and maximum  
Reference range  

N = patients  FA = fatty acids  AAM = amino acid mixture
Bone Mass Density and Bone Turnover Markers

BTM were above reference range for resorption marker CTx (21/56; 20/43 children and 1/13 adults) and formation marker PINP (42/56; all 13 adults and 28/43 children) (Figure 7). Mean Z-scores for lumbar, femoral and hip BMD were overall normal with Z-scores below -2 in 4.9% (n=2/41), 7.4% (n=2/27) and 5.9% (n=2/34) of patients respectively (Figure 8). No differences in BMD or BTM were found based on BH4 use or severity of disease.

Median physical exercise for adults was 205 min/week, for children 12-17 years 325 min/week and those 1-11 years 180 min/week. A total of 25 patients (41.7%) have suffered a fracture. Fifteen patients suffered a single fracture episode, 9/25 two and 1/25 three. All fractures were caused by compatible trauma and healed without complications. One patient had a positive fracture history as defined by the ISCD but BMD was within normal range.

Multivariable linear regression

One of our aims was to use multivariable linear regression to investigate associations between blood levels, BMD, BTM and intake. However, because assumptions needed to justify such a model were not met (the data of the dependent variable was not distributed in a straight-line), we do not report results concerning associations between intake and serum levels of micronutrients, nor between serum levels of micronutrients and bone health.
Figure 7. Bone turnover markers
Median, minimum and maximum  
Reference range  

N = patients
PINP = resorption marker procollagen type I N-terminal propeptide
CTX = formation marker carboxy-terminal collagen crosslinks

~ 160 ~
**Figure 8.** Bone mineral density (BMD)
Median, minimum and maximum ————
Reference range ————————

N = patients
DISCUSSION

We evaluated dietary intake and deficiencies of micronutrients and essential FA, BMD, BTM, physical activity and fracture history in a large group of patients with PKU. In spite of the complex diet with many natural food products replaced by designated AAM and low protein foods, most blood levels of micronutrients are in the normal range. Some exceptions need to be addressed specifically.

**Micronutrients**

*Vitamin D*

Serum 25-OH vitamin D2+3 levels were below reference range in 14% of patients, fully comparable to individuals in the general population in whom concentrations below reference range are also frequently observed [18]. However, 2 patients showed levels in the range associated with clinical symptoms (<25 nmol/L [18]). For this reason, despite near normal bone health outcomes (BMD, fracture risk and BTM), it may be advisable to yearly evaluate intake and determine blood levels, and to supplement patients when levels are <50 nmol/L [18,19].

*Zinc*

Zinc serum levels were below reference range in 14% of patients, despite an intake above SAR in 52% of patients. This finding has been reported before [4,6]. As a low intake of animal protein in a vegetarian diet may decrease absorption of zinc [20], it seems probable that the severe restriction of natural protein in the PKU diet decreases zinc absorption. Zinc deficiency is associated with abnormalities in growth, sexual maturation, wound healing, hair loss, visual dark adaptation and anorexia [21]. Hair loss has been described at serum levels below 11 µmol/L (72 µg/dL) [22] and skin lesions below 9.2 µmol/L (60 µg/dL) [23]. The clinical relevance of our findings is debatable and further studies need to be done on how to effectively increase zinc uptake in PKU patients. Especially considering the fact that a large proportion of our patients already have intakes exceeding SAR, and that to achieve an increase of 6% of serum levels intake needs to be doubled [24].
Selenium

Low plasma selenium serum levels have previously been described in PKU [6]. In our cohort dietary intake was below reference in 41% and serum levels in 46% of patients. Selenium is best absorbed when ingested as an organic form while inorganic forms, such as sodium selenite used in AAM, are less well absorbed. Selenium deficiency may lead to cardiomyopathy, depressive symptoms or osteoarthropathy [25-27]. Mood disorders and depressive symptoms have previously been described in PKU [28]. An increased risk for depressive symptoms has been reported at serum levels of <1.04 µmol/L (82 µg/L) [26], which is above the lowest reference range of normal (0.8 µmol/L) in the present study. Because intake in many patients is low it seems advisable to annually evaluate intake and blood levels and consider supplementation if levels are below advised reference ranges (supplementing up to 400 µg/day is considered to be safe in adults [29]). However, no large studies are available concerning the clinical relevance of lowered selenium levels. Therefore, larger studies are indicated to further assess the clinical relevance of our findings.

Folic acid

Folic acid intake and blood levels were remarkably high in patients using AAM. Five patients showed intake levels above SAR (Figure 1). Because all patients off diet showed serum levels in the normal range, elevated serum levels appear to be due to the fortified AAM [30]. As there is discussion on the safety of high levels [31] it deserves due consideration to lower folic acid amounts in AAM. Our findings are confirmed by the study by Stolen et al reporting similar results [32]. Magnesium, vitamin B6 and B12 are elevated in dietary intake and blood levels. However as intakes are within SAR and these micronutrients are not known to be toxic adaptation of intake may not be warranted [13].

Amino acids

Plasma arginine (a conditionally essential amino acid) was below reference range in 19% of patients. This decrease cannot be assigned to hemolysis (when arginase converts arginine to ornithine) as ornithine levels were normal. Arginine is important for many
cellular functions, including nitric oxide production and urea cycle function [33,34]. Glutamine levels were fully normal demonstrating normal function of the urea cycle. Further research is necessary to determine whether it may be advisable to increase supplementation of arginine in AAM.

Plasma tyrosine was low in our patients, especially in children and adolescents. Patients who fasted overnight had lower median tyrosine levels (40 umol/L) than those fasting 3-6 hours (54 umol/L), confirming previous reports of low fasting tyrosine values in PKU [35]. AAM use has been demonstrated to cause large fluctuations in plasma tyrosine over the day and effects of extra tyrosine supplementation on outcome are yet unclear [36]. Further investigations on effects of intake on plasma levels are warranted.

Asparagine (a non-essential amino acid) levels were low in 1/3 of patients, but not much is known about implications of deficiency. Asparagine is not supplemented in AAM. Further examination is needed to objectify if asparagine deficiency is clinically relevant, and if it should be added to AAM.

2-Aminobutyric acid is formed from methionine and threonine [37], and was low in our patients. Low plasma levels have been reported before in a small patient sample (n=4) [37]. It is unclear if decreased levels have a clinical effect.

Hydroxyproline (a bone resorption marker) is solely elevated in adolescents, representing increased protein turnover in bone during growth [38].

**Erythrocyte fatty acids**

Essential FA are precursors of thromboxanes, leukotrienes and prostaglandins [39]. DHA and EPA are known to have cardio-protective effects [40] and deficiencies may lead to CNS disease (specifically DHA). Deficiencies of EPA and AA may affect the immune system [39,39].

Our patients had normal to elevated levels of LA and it's metabolites. This can be explained as both LA and arachidonic acid are supplemented in AAM.

Of the ALA metabolites EPA and DHA are frequently supplemented in AAM. Perhaps EPA is insufficiently supplemented as it is below reference levels in 6% of our patients, and for this reason it may be considered to increase EPA supplements in AAM. DHA is
more often and higher supplemented in AAM, explaining why levels are high compared to the reference range in our patients. Significantly lower levels of DHA and EPA are found in patients using AAM without FA versus those using FA supplemented AAM. For this reason it may be advisable to prescribe FA containing AAM or warrant sufficient intake with other means of supplementation.

**Bone health**

In our population 4.9% and 7.4% of patients had a lumbar and femoral BMD Z-score ≤ -2 respectively. This finding is in line with data reported in a previously published meta-analysis by our study group [8]. None of our patients had osteoporosis as defined by the ISCD and the lifetime fracture prevalence of patients with PKU seems comparable to the age-standardized lifetime fracture prevalence of the general population in England (41.7% versus 38.2%) [41].

Physical exercise in adults met the WHO recommended 150 min/week. Of children aged 12-17 years 80% did not meet the recommended 60 min/day of exercise, which is comparable to the general population [42]. We have no reason to believe that insufficient physical activity in this patient group has a negative effect on bone health other than it would in the general population.

BTM are remarkably elevated, formation more than the resorption marker. Implications of these findings are yet unclear and outcomes of other studies have shown contradictory results with elevated, decreased or even normal BTM in patients [8]. We hypothesize that this disbalance of bone turnover in our population may have effect on bone health when patients are over the age of 50 years. However, in the light of the near normal BMD and fracture prevalence in this patient group the clinical implications of these findings are yet unclear and need further study.
Chapter 6

*Multivariable linear regression*

Unfortunately we were not able to properly investigate associations between blood and intake levels of assessed nutrients, nor between outcomes of researched nutrients and BMD, BTM. However, because these relations are of great interest to achieve further knowledge on the etiology of nutrient deficiencies and bone health, larger cohort or case-control studies are indicated.

**CONCLUSIONS**

In conclusion, we detected lower levels of several micronutrients and FA in this sample of patients with PKU. However, specific complications that may be related to these alterations, other than BMD, were not assessed in this study. Those micronutrients that have been studied in large cohorts as potentially leading to risk (e.g. vitamin D and selenium) could be considered to be supplemented. At this time there is no convincing evidence for supplementation of the other nutrients.

Furthermore, although fracture prevalence is normal, a slightly more prevalent low BMD and elevated BTM are found. The clinical implications may be limited as none of the patients have osteoporosis as defined by the ISCD. However, the meaning of these outcomes is yet unclear and follow up into older age is warranted.
REFERENCES


